Department of Defense Legacy Resource Management Program

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Brown treesnake pheromone attractant for assessing large-scale population control and incipient population detection

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Background

The larger goal for this project was to determine whether the sex pheromone of the brown treesnake would be an effective tool for 1) assessing efficacy of large-scale eradication operations on Guam, and 2) detecting small newly established populations on nearby islands at risk, such as Tinian and Saipan. Large-scale eradication of snakes in the areas surrounding military lands is the only practical approach for potentially reducing the need for snake interception efforts currently conducted in and around areas of transport. Large-scale eradication also requires methods to determine efficacy of the operation. The primary methods available for these assessments are snake traps containing a live mouse and bait tubes containing a dead mouse. A sex pheromone attractant, however, has the potential to be a more potent attractant than a food bait. Coming with the projected expansion of military activities on Guam and associated islands is a heightened risk of inadvertently transporting the brown treesnake to other sensitive locations. Detection of small incipient populations at such locations may best be accomplished using a sex pheromone because such populations, by nature, tend to establish in food -rich locations. Snakes in food-rich areas may not be very trappable, and are more likely to respond to the sex pheromone than food baits. A tool for detection of incipient populations is needed so they can be extirpated before becoming established. The pheromone is the female sex pheromone, thus it likely attracts only males. Practical operational use of the sex pheromone hinges on several factors -

1) Pheromone must have at least equal, or longer, range than current attractants.
2) Pheromone must be at least equal, or more attractive, to snakes than mouse attractants.
3) Pheromone must be relatively easy and economical to acquire and use in the field.
4) Pheromone must have good storage stability.
5) Longevity of pheromone under field conditions must at least equal those of current attractants.

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Methods

Objective 1. Determining distance males will follow pheromone trail - Work under this Objective and Objective 2 (below) was conducted in the field on Guam. Live adult male BTS were uniquely marked using implanted microchips, and then held in captivity until testing. Guidelines (20-100m long) were suspended along roadsides. Each guideline passed through hoop readers of AVID microchip tag readers placed at the beginning and end of a guideline and at regular intervals in between. Cages (snake traps modified so that a door can be affixed open), each housing a microchipped male, were placed at one end of a guideline and the occupant allowed to acclimate for 1-2 days before testing (figure 1). The opposite end of the guideline lead into a trap that could not be exited. Cameras equipped with infrared illumination connected to video recorders with real time recording capability were placed to record snake movement at various sections of a guideline. Female skin secretions collected under Objective 4 (below) were resuspended in 1-2 mL of solvent and sealed in glass vials before use. On the day of a test, skin secretion-solvent mixture was applied to a guideline. On each test day there was also a control guideline treated only with solvent. Scientists at NWRC are currently testing techniques for applying pheromone to guidelines that should enable a male to determine a female 's direction of movement. On the day of a test, the cage door was opened, and the end of the pheromonally-infused guideline (or control) passed inside. It was anticipated that males will in most cases exit their holding cage that evening and encounter the guideline. Males that follow a guideline would pass through hoop readers where male identity and time of passage wouldl be recorded. At least 30 trials were to be conducted using a different male and fresh guideline each trial. Metrics to be determined for each trial would include each distance interval along guideline passed by a male, and whether male enters trap at guideline end. Males captured in traps were to be scanned with reader to confirm identity. These data would be be used to determine the fraction of males traversing each section of guideline and percent trap catch. Any non -test (wild) snakes that are caught would also be examined.

The possibility that the brown treesnake pheromone may become airborne and thus detectable to males at a distance was also evaluated. Home cages of 13 vitellogenic and 13 non-vitellogenic females were placed within an enclosure on Guam into which 15 adult male brown treesnakes were released (figures 2 and 3). Cage tops and bottoms were covered with ¼ in wire hardware cloth and were thus highly ventilated. Cages were arranged on the ground

under forest canopy in two parallel rows about 60 cm apart, with vitellogenic females in one row and nonvitellogenic females in the other. A video camera system was placed at the head of the aisle between the two rows such that snake activity at cages was monitored. It was hypothesized that if the female pheromone were airborne, males would appear at the cages of females and attempt to gain entrance to cages of interest.

Objective 2. Comparison of pheromone vs. mouse attractant - Methods were to follow those described in Objective 1, but with the following modifications. Standard WS snake traps baited with a live mouse bait were placed parallel to guidelines (1-2 m distant). Cameras would be directed at these traps. In addition to those metrics recorded under Objective 1, identity of a male captured in a mouse-baited trap and the trap's linear position alongside guideline were recorded. These data were used to determine the percent trap catch for each interval of guideline. Any non -test snakes that were caught would also be examined.

Objective 3. Collection of pheromone from wild-caught snakes - Adult female snakes were obtained from WS Guam. After euthanasia, skin were extracted by placing carcasses in three successive baths of chloroform-methanol (2-1, 1-1, 1-2) for 2 h each (figure 4). Skin extracts were combined and concentrated by rotary evaporation, evaporated to dryness under a stream of nitrogen, desiccated in vacuo, and then weighed. Samples were stored in glass vials evacuated with nitrogen, sealed, and then stored at -12°C. The number of adult females caught by WS during one month was determined and together with the mass of skin lipids obtained per female, the estimated monthly collection of skin lipids was calculated.

Objective 4. Storage Stability - Samples collected in Guam were returned to NWRC facilities for processing and analysis. The samples were subdivided and one aliquot was spiked with the surrogate nonadecanone at a concentration of approximately 20 ppm. The samples were sealed in vials under nitrogen and stored at -12°C until analyzed by GC-MS. The integrity and stability of the sample was evaluated based on the recovery of the surrogate and the variance between the replicates from the subdivided samples. Samples were analyzed monthly for the first three months and every three months thereafter for a year. Samples were monitored quarterly after the first year. In addition, a male BTS-guided bioassay developed at NWRC was

used to evaluate sample integrity at the outset of this work, and at selected times thereafter. Bioassay work was provided as in-kind.

Results and Discussion

Field tests were conducted in a forest setting using experimental guidelines equipped with camera and PIT tag reader systems to monitor behavior and movements of males experimentally released onto guidelines. All releases were ‘soft’ releases using males collected by USDA Wildlife Services and then held singly in outdoor cages for at least one week prior to release. Soft releases involved transporting each male to the release site within a hide tube it had been accustomed to in the late afternoon on date of release. Tubes were placed on release racks alongside guidelines inoculated throughout with either female skin lipid secretions or vehicle carrier. Hide tubes were opened during the evening hours and males allowed to exit onto guidelines as they chose. We were unsuccessful at meeting Objectives 1 and 2. Male brown tree snakes did not leave hide tubes slowly and investigate guidelines treated with the female skin secretions as expected. Instead, upon release, all male brown tree snakes escaped the testing site and did not return. The assessment was that behavior and perhaps sexual function of males, after being brought into captivity, is unduly disrupted.

Lesson Learned: All further testing should be done either using males well-acclimated to a laboratory setting or wild males on Guam.

During the one night of testing to see if the pheromone was airborne, no males appeared at cages of any females, even though skin secretions of at least some of the vitellogenic females had been recently been proven attractive to males (concurrent experiments). This experiment was conducted 14 days after the male snakes were released into the enclosure. If the pheromone does have airborne bioactivity, it stands to reason that some males should have been attracted because the un-naturally large number of vitellogenic females assembled in the enclosure had the potential to produce an ultra-strong pheromone signal. Regardless, the possibility of an airborne mechanism should receive some additional inquiry. Upon completion of the experiment, all vitellogenic females were euthanized and the ovaries dissected out to

confirm states of follicular development. All 13 females had fully enlarged ovarian follicles (Figure 7). However, follicles in three females appeared to be undergoing atresia.

Objectives 3 and 4 have been partially met. Female skin lipid yields were determined from mature females captured during a 30-day period using all traps in current operational use by USDA Wildlife Services (~3000). A total of 319 snakes were captured and examined. Of these, 42 females were unequivocally sexually mature (but non-reproductive), and 9 were reproductive (containing enlarging ovarian follicles). A series of solvent washes were used to collect skin lipids from skin surfaces of 26 non-reproductive and 6 reproductive females producing total dry lipid yields yielding of 782 and 176 mg, respectively, or approximately 30 mg of lipids per carcass (figure 5). Initial GC/MS analysis of the skin lipids from two females reported three main components: Phthalate (largest peak, a plasticizer), cholesterol, and C16/C18 fatty acid chains.

The cholesterol extract may contain the sexual pheromone. Lathosterol (5&agr;-Cholest-7-en-3β-ol), a cholesterol precursor, was recently isolated from brown African house snakes (*Lamprophis fuliginosus*) and was shown to increase male snake sexual behavior toward juvenile females as compared to a cholesterol control (Wilmes 2012). Wilmes 2012 found that lathosterol pheromone trails were strongly followed by males in “Y” mazes for one week and the trails faded after 2 weeks. Confoundingly, male snakes also trailed other male snakes within the Y mazes.

Storage stability tests paired with male bioassays have not yet been conducted on the collected skin lipids to determine chemical stability and duration of bioactivity. If bioactivity can be retained under storage conditions, ‘harvest’ of pheromone, even from the relatively few reproductive females caught per year (estimated at ~70/yr), would likely meet the operational needs of a pheromone-based detection tool.

Recommendations.

1. Considering the results from this experiment, a future experiment could be conducted to evaluate the potential application of the attractiveness of snake pheromones. In a

natural setting on Guam, guide ropes could be attached to baited and unbaited snake traps. Half of the guide ropes would be treated with female skin lipid secretions or vehicle carrier alone (may need several solvent extracts). The number and sex of snakes trapped would be compared to provide a relative effectiveness of the method. This experiment would also aid in determining if this line of research had potential utility as an attractant tool for trapping snakes in field conditions. If this method increased trap efficacy significantly, future work would be warranted.

2. Further work on the storage stability of the compounds identified would likely not be useful at this time. Until the female pheromone(s) responsible for the male response is identified, the stability of the compound cannot be tested.

Literature Cited

Wilmes, Anthony J. Pheromone trailing, pheromone trail decay, and identification of the female sex pheromone in the African brown house snake Lamprophis fuliginosus. Diss. SAINT LOUIS UNIVERSITY, 2012.

Figure 1. Experimental as-built soft-release stations, rope guidelines, PIT tag readers and traps for testing male brown tree snake pheromone-trailing in Guam.

Figure 2. Home cages of vitellogenic and non-vitellogenic female brown treesnakes (Boiga irregularis) placed for one night within outdoor enclosure containing free-ranging male brown treesnakes and filmed overnight.

Figure 3. Snake enclosure at Guam National Wildlife Refuge

Figure 4. Extraction of skin lipids from female brown treesnake carcasses using solvent washes.

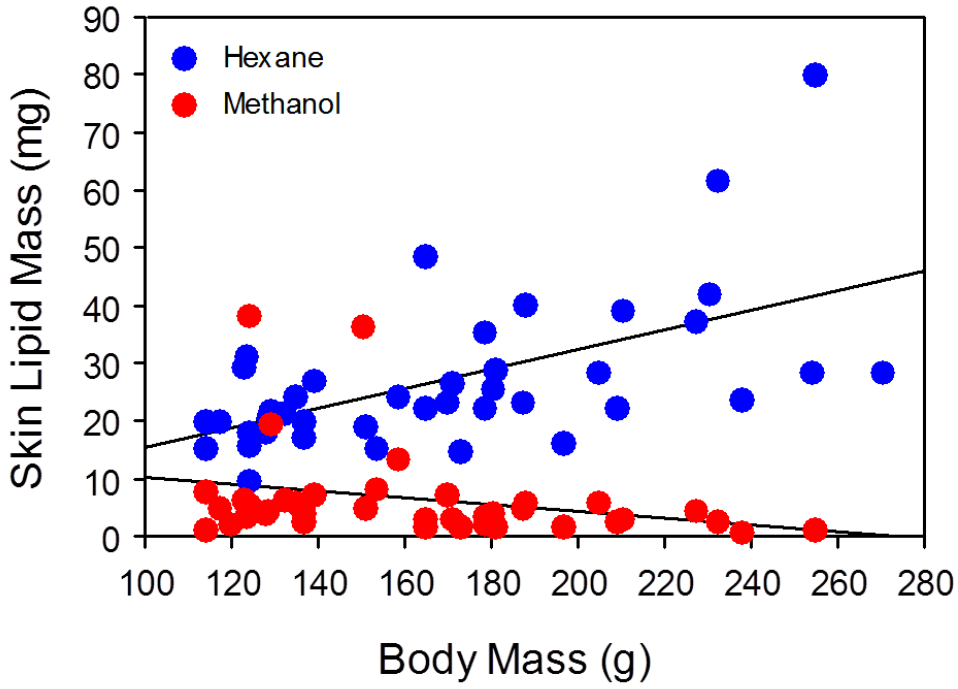

Figure 5. Skin lipids (mg) extracted from female BTS carcasses via methanol and hexane washes.

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